AMP-activated protein kinase regulates the assembly of epithelial tight junctions

Li Zhang*, Ji Li†, Lawrence H. Young†‡, and Michael J. Caplan*‡§

*Department of Cell Biology, †Department of Internal Medicine, Section of Cardiovascular Medicine, and ‡Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06520

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AMP activated protein kinase (AMPK), a sensor of cellular energy status in all eukaryotic cells, is activated by LKB1-dependent phosphorylation. Recent studies indicate that activated LKB1 induces polarity in epithelial cells and that this polarization is accompanied by the formation of tight junction structures. We wished to determine whether AMPK also contributes to the assembly of tight junctions in the epithelial cell polarization process. We found that AMPK is activated during calcium-induced tight junction assembly. Activation of AMPK by 5-aminoimidazole-4-carboxamide ribonucleoside facilitates tight junction assembly under conditions of normal extracellular Ca^{2+} concentrations and initiates tight junction assembly in the absence of Ca^{2+} as revealed by the relocation of *zonula occludens* 1, the establishment of transepithelial electrical resistance, and the paracellular flux assay. Expression of a dominant negative AMPK construct inhibits tight junction assembly in MDCK cells, and this defect in tight junction assembly can be partially ameliorated by rapamycin. These results suggest that AMPK plays a role in the regulation of tight junction assembly.

AMPK | ZO-1

AMPK acts as a sensor of cellular energy status in all eukaryotic cells. It is a heterotrimer, composed of a catalytic α-subunit complexed with β- and γ- regulatory subunits (1). Its kinase activity is stimulated by elevated AMP/ATP ratios. The binding of AMP to its γ-subunit makes AMPK a better substrate for the upstream kinases that phosphorylate the AMPK α-subunit at T172 (2). In general, activation of AMPK switches on ATP-generating pathways and switches off ATP-consuming processes.

In recent studies AMPK was reported to be activated by LKB1-dependent phosphorylation (3–5). The tumor suppressor gene LKB1 was cloned through a linkage analysis of Peutz-Jeghers syndrome patients (6, 7). It has been shown that LKB1 is a negative regulator of the mammalian target of rapamycin (mTOR) signaling pathway through the sequential activation of AMPK and of the tuberous sclerosis complex (TSC)1/TSC2 tumor suppressor complex (8–10).

Previous genetic studies performed in *Caenorhabditis elegans* (11) and *Drosophila melanogaster* (12) indicated that the LKB1 homologs partioning-defect (PAR)4 and dLKB1, respectively, play essential roles in directing cell polarization during embryogenesis in these organisms. A recent study demonstrates that activated LKB1 is capable of mediating junction formation and polarized protein sorting in single intestinal epithelial cells, independent of any requirement for cell–cell contact (13). These studies indicate that LKB1 plays an important role in regulating cell polarity and suggest that mutations in LKB1 may induce tumor formation, at least in part, as a result of the loss of cell polarity.

The mechanism through which LKB1 participates in regulating cell polarity is not well understood. A series of studies have shown that this process involves the phosphorylation of microtubule-affinity-regulating kinase (MARK), a PAR1 homolog, by LKB1 (14). This phosphorylation event permits MARK to participate in interactions with other PAR homologs (15–20). A recent study, however, suggested that the regulation of cell polarity is independent of the kinase activity of LKB1 (21). In this study, mutations of LKB1 that did not disrupt LKB1 kinase activity significantly reduced LKB1-mediated activation of AMPK and compromised the ability of LKB1 to establish and maintain polarity in both intestinal epithelial cells and migrating astrocytes. It appears likely, therefore, that the establishment and maintenance of cell polarity are linked, at least in part, to AMPK activation.

The tight junction is a polarized structure that forms a seal at the superior aspect of the lateral surface of plasma membrane when epithelial cells acquire polarity. Tight junctions regulate the passage of ions and small molecules through the paracellular pathway (22). Tight junctions also restrict the diffusion of membrane proteins between the apical and basolateral compartments (23). This latter function of tight junctions appears to participate in the maintenance of epithelial cell polarity under normal circumstances.

We report that AMPK regulates tight junction assembly in the MDCK line of polarized canine renal epithelial cells. We show that AMPK is activated during calcium-induced tight junction assembly. We also show that the assembly of tight junctions is facilitated by the up-regulation of AMPK activity and is inhibited by the down-regulation of AMPK activity. We further show that direct inhibition of mTOR by rapamycin partially ameliorates the defects in tight junction assembly caused by loss of AMPK activity, providing a potential pathway through which AMPK may regulate tight junction assembly.

Results

AMPK Is Activated During Calcium-Induced Junction Assembly. Extracellular calcium is essential for the assembly of cell junctions (24, 25). Cell–cell junctions in polarized epithelial cells are lost rapidly when calcium is withdrawn from the medium, and readdition of calcium (i.e., calcium switch) to the medium can reinitiate the assembly of both tight junctions and adherens junctions (26). To investigate the role of AMPK in the process of tight junction assembly, we used MDCK epithelial cells as our model system, and the assembly of tight junctions in this cell line

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Abbreviations: 2-DG, 2-deoxy-D-glucose; ACC, acetyl coenzyme A carboxylase; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AMPK, AMP-activated protein kinase; CaMKK, calcium/calmodulin-dependent protein kinase kinase; DN, dominant negative; HCM, high-calcium medium; LCM, low-calcium medium; MARK, microtubule-affinity-regulating kinases; mTOR, mammalian target of rapamycin; PAR, partitioning-defect; TER, transepithelial electrical resistance; TSC, tuberous sclerosis complex; ZO-1, zonula occludens 1.

§To whom correspondence should be addressed at: Department of Cellular and Molecular Physiology, Yale University School of Medicine, P.O. Box 208026, New Haven, CT 06520-8026. E-mail: michael.caplan@yale.edu.

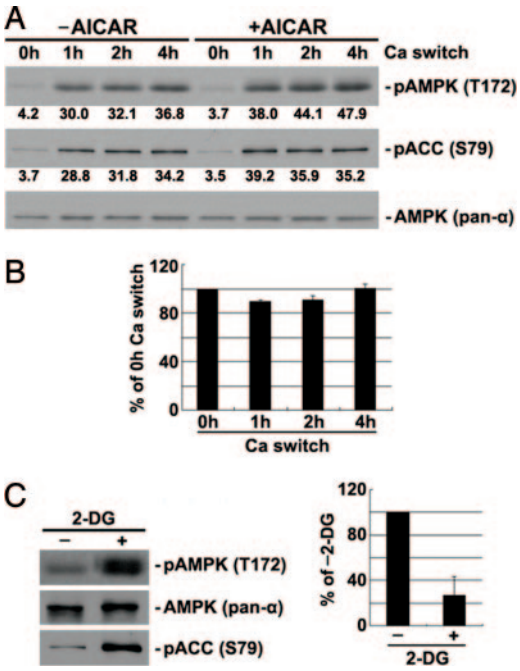

Fig. 1. AMPK is activated during a calcium switch. (*A*) MDCK cell lysates from the indicated time points after the switch from LCM to HCM in the absence or presence of AICAR were blotted with the indicated antibodies. The relative intensity of the bands in arbitrary units appears below each lane. (*B*) The ATP content of lysates from cells subjected to a calcium-switch protocol was measured. The ATP concentrations are represented as percentages of those found in cells maintained in LCM, which are set to 100%. Data were from three experiments. (*C Left*) Lysates from cells incubated with or without 2-DG were blotted with the indicated antibodies. (*C Right*) Cell lysates from 2-DG treated cells were subjected to an ATP assay. The ATP concentrations are represented as a percentage of those found in cells cultured under normal conditions, which are set to 100%. Data were from three experiments.

was manipulated by switching culture conditions between low-calcium media (LCM) and high-calcium media (HCM).

To examine AMPK phosphorylation during calcium switch, cell lysates from 1 h, 2 h, and 4 h after the switch from LCM to HCM were blotted with an antibody specifically recognizing AMPK phosphorylated at T172. The level of AMPK phosphorylation was elevated at each of these time points compared with that detected in cells maintained in LCM (Fig. 1*A Top*, −AICAR). The total amount of AMPK, however, remained unchanged, as shown in a blot probed with an antibody recognizing both phosphorylated and unphosphorylated AMPK (Fig. 1*A Bottom*, −AICAR).

The kinase activity of AMPK depends on T172 phosphorylation (2). We next examined, therefore, whether the AMPK kinase activity is increased by calcium switch. Acetyl CoA carboxylase (ACC) is a well documented substrate for AMPK, and the extent of its phosphorylation reflects the *in situ* level of AMPK activity (27). We observed an 8-fold increase of ACC phosphorylation (Fig. 1*A Middle*, −AICAR) after the readdition of calcium, representing an increase in AMPK activity. The levels of both phosphorylated AMPK and phosphorylated ACC remained stably elevated for up to 16 h and then gradually subsided by the 24-h time point after the readdition of calcium (data not shown). Taken together, these data demonstrate that AMPK is activated during calcium-induced junction assembly.

Because the activation of AMPK might be attributable to changes in cellular energy status that might perhaps be induced by the calcium-switch protocol, we next examined the cellular ATP levels in MDCK cells that were maintained in LCM or that had been switched for 1 h, 2 h, and 4 h to HCM. We observed only very minor changes in cellular ATP levels associated with calcium switch (Fig. 1*B*). As a control experiment, we treated MDCK cells for 1 h with 25 mM 2-deoxy-D-glucose (2-DG) (Sigma), which promotes AMPK activation by blocking glucose transport and phosphorylation, thus reducing ATP production. We observed that both AMPK phosphorylation and AMPK activity increase with 2-DG treatment to an extent comparable to that found in the cells undergoing calcium switch (Fig. 1*C Left*). In contrast, however, 2-DG treatment induces a dramatic decrease in the cellular ATP level (Fig. 1*C Right*). Evidently, the AMPK activation observed in cells subjected to the calcium-switch protocol is not attributable to corresponding changes in cellular ATP levels.

Activation of AMPK by AICAR Facilitates Tight Junction Assembly. AICAR is rapidly transported into cells and phosphorylated to form the AMP mimetic *S*-aminoimidazole-4-carboxamide ribonucleoside monophosphate (ZMP), which activates AMPK without changing the intracellular levels of AMP or ATP (28, 29). We treated MDCK cells with 1 mM AICAR for 2 h to activate AMPK. As expected, we found that this treatment led to increased levels of AMPK and ACC phosphorylation (Fig. 2*A Left*). These data serve to confirm the activation of AMPK by AICAR in the MDCK cell system.

We first compared the elevation of AMPK phosphorylation and activity in MDCK cells during calcium switch in the presence or absence of AICAR. We observed very minor differences between the two conditions, although the levels of both phosphorylated AMPK and phosphorylated ACC are slightly higher in the presence of AICAR than in the absence of AICAR. It appears that the calcium-switch-induced activation of AMPK was not enhanced further by simultaneous AICAR treatment (Fig. 1*A*).

To characterize morphologically the rate of calcium-induced tight junction assembly, we monitored the time course of the relocation of a tight junction protein, zonula occludens 1 (ZO-1), to the cell–cell junctions after the readdition of calcium. We observed that ZO-1 relocation is accelerated significantly in the cells incubated in the presence of AICAR during the calcium switch. At the 2h time point, the amount of ZO-1 associated with the cell–cell junctions is twice as much as that detected in cells incubated in the absence of AICAR (Fig. 2 *B* and *C*).

We next monitored the establishment of transepithelial electric resistance (TER) in MDCK monolayers during the calcium switch. We did not detect significant differences at the early time points in this process, regardless of the presence of AICAR. At later time points, however, the TER reached a higher peak value in the MDCK monolayers cultured in the presence of AICAR compared with those cultured in the absence of AICAR (Fig. 2*D*), indicating that the establishment of TER is promoted by AICAR treatment. We also measured the paracellular flux of 70-kDa Texas red-conjugated dextran in MDCK monolayers during the calcium switch in the presence or absence of AICAR. We did not observe major changes in the flux rate induced by the treatment with AICAR. At the 1 and 2h time points, however, the presence of AICAR led to a slight but statistically significant decrease in the dextran flux rate across the monolayers (Fig. 2*E*).

We also pretreated MDCK cells with AICAR in LCM to activate AMPK before the calcium switch. We confirmed that in LCM, AICAR is also capable of activating AMPK (Fig. 2*A Right*). We observed similar acceleration of ZO-1 relocation during the calcium switch following this treatment (Fig. 2*C*). We were surprised to notice that in the presence, but not in the absence, of AICAR, the relocation of ZO-1 to cell–cell junctions could be initiated even in LCM. We observed fragmented strands of ZO-1 staining at cell borders with 1h of AICAR incubation. The morphological organization of these fragmen-

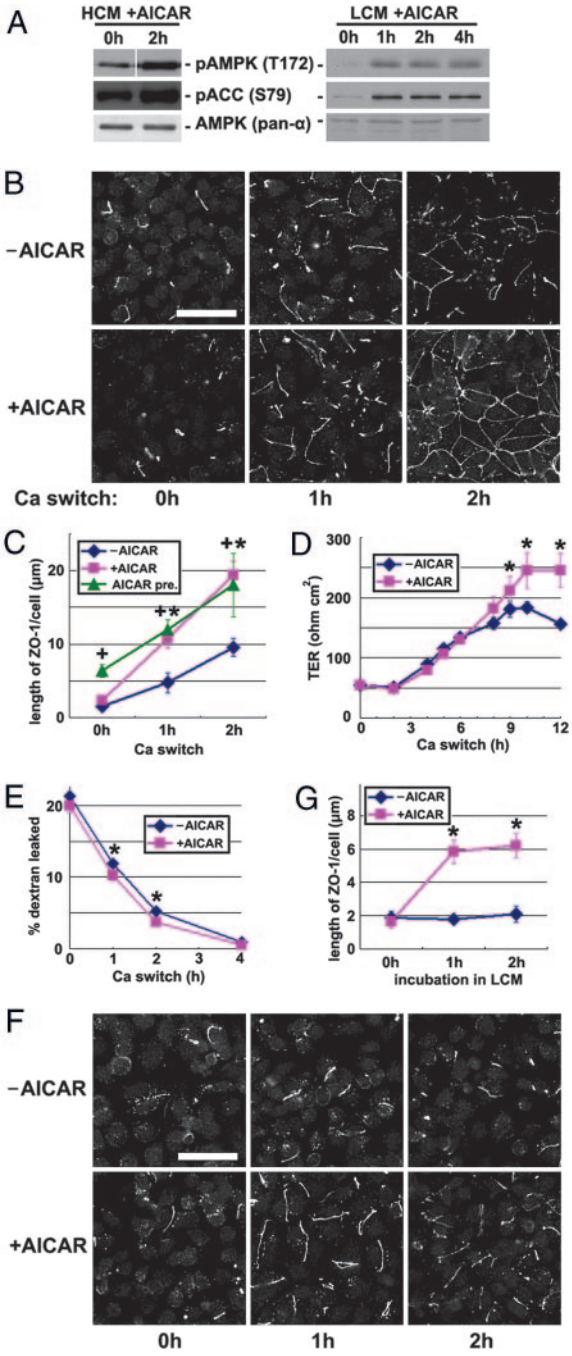

Fig. 2. Activation of AMPK by AICAR facilitates tight junction assembly. (*A*) Lysates from MDCK cells maintained in HCM or LCM and incubated with AICAR for the indicated times were blotted with the indicated antibodies. (*B*) Localization of ZO-1 in cells subjected to a calcium switch with AICAR absent or present in the HCM. (Scale bar: 30 μm.) (*C*) Quantification of ZO-1 relocation to cell–cell junctions in the absence or presence of AICAR or with AICAR pretreatment. The asterisks denote significant differences in the presence vs. the absence of AICAR; the crosses denote significant differences between AICAR pretreatment vs. the absence of AICAR by Student's *t* test ($P < 0.05$). (*D*) TER of cell monolayers subjected to a calcium switch with AICAR absent or present in the HCM. The asterisks denote significant differences in the presence vs. the absence of AICAR. (*E*) Paracellular flux assay performed on cell monolayers subjected to a calcium switch with AICAR absent or present in the HCM. The asterisks denote significant differences detected in the presence vs. the absence of AICAR. (*F*) Localization of ZO-1 in cells maintained in LCM in the absence or presence of AICAR. (Scale bar: 30 μm.) (*G*) Quantification of ZO-1 relocation to cell–cell junctions in LCM. The asterisks denote significant differences detected in the presence vs. the absence of AICAR by Student's *t* test ($P < 0.05$).

tary ZO-1 strands, however, did not continue to mature following further AICAR incubation in LCM (Fig. 2 *F* and *G*) and finally disappeared by 4 h (data not shown).

Expression of Dominant Negative (DN) AMPK Inhibits Tight Junction Assembly. AMPK is a heterotrimer that requires all three subunits to be functionally active (30). Overexpression of a mutant "kinase-dead" α-subunit induces a DN effect because it competes with its native counterpart for incorporation into the complex (31).

To investigate the effect of AMPK inhibition on tight junction assembly we generated Tet-Off MDCK stable cell lines that inducibly overexpress a kinase-dead (K45R) construct of the AMPK α2 subunit. We were able to track the replacement of the predominant native α1 subunit by its K45R α2 counterpart by using AMPK α1- and α2-specific antibodies. The expression of the K45R α2 subunit is modulated by doxycycline (Fig. 3*A*). We observed that in cells cultured in the absence of doxycycline, the DN AMPK is expressed, and the levels of both AMPK phosphorylation and activity were inhibited, as shown by the blot probed with anti-pAMPK and anti-pACC (Fig. 3*B*).

We found that as a consequence of the expression of DN AMPK, the elevation of AMPK phosphorylation and activity during calcium switch was substantially suppressed. This behavior stands in sharp contrast to the significant calcium-switch-induced elevation of both AMPK phosphorylation and activity observed in the same cells cultured in the presence of doxycycline, which effectively suppresses the expression of the DN AMPK construct (Fig. 3*C*).

We next used these cell lines to investigate the effects of AMPK inhibition on tight junction assembly. During steady-state culture no obvious defect in tight junction morphology could be observed with the expression of DN AMPK (data not shown). However, substantially different behaviors were observed during the calcium switch. In contrast to the normally rapid ZO-1 relocation to cell–cell junctions found in the cells cultured in the presence of doxycycline, we observed that in the cells cultured in the absence of doxycycline, the relocation of ZO-1 was significantly delayed (Fig. 3 *D Top* and *Middle* and *E*). Ultimately, however, the morphological correlates of the tight junctions were slowly but apparently completely formed in the cells cultured in the absence of doxycycline. These results were confirmed in two independent K45R AMPK α2-expressing cell lines.

The establishment of TER appears to be normal in transfected MDCK cell monolayers cultured in the presence of doxycycline when the DN AMPK construct is not expressed. In cell monolayers cultured in the absence of doxycycline, however, the establishment of TER is substantially inhibited (Fig. 3*F*). Following the readdition of calcium, the resistance values of monolayers cultured in the absence of doxycycline are significantly lower than those cultured in the presence of doxycycline. The paracellular flux was also affected by the expression of the DN AMPK construct. We observed a significantly higher flux rate in the MDCK monolayers cultured in the absence of doxycycline (when the DN AMPK construct is expressed) than in those cultured in the presence of doxycycline (when DN AMPK construct is not expressed) (Fig. 3*G*), indicating that the expression of the DN AMPK construct increases paracellular permeability.

Rapamycin Partially Ameliorates the Delay of Tight Junction Assembly Caused by DN AMPK. AMPK is a critical component of a pathway that links cellular energy status with cell proliferation. Activated AMPK activates TSC, leading to the inhibition of mTOR and, thus, to the down-regulation of cell growth and proliferation (8–10). Rapamycin directly inhibits mTOR. Thus, if the inhibition of mTOR by rapamycin can correct the defects in junction assembly caused by DN AMPK, it suggests that AMPK's regu-

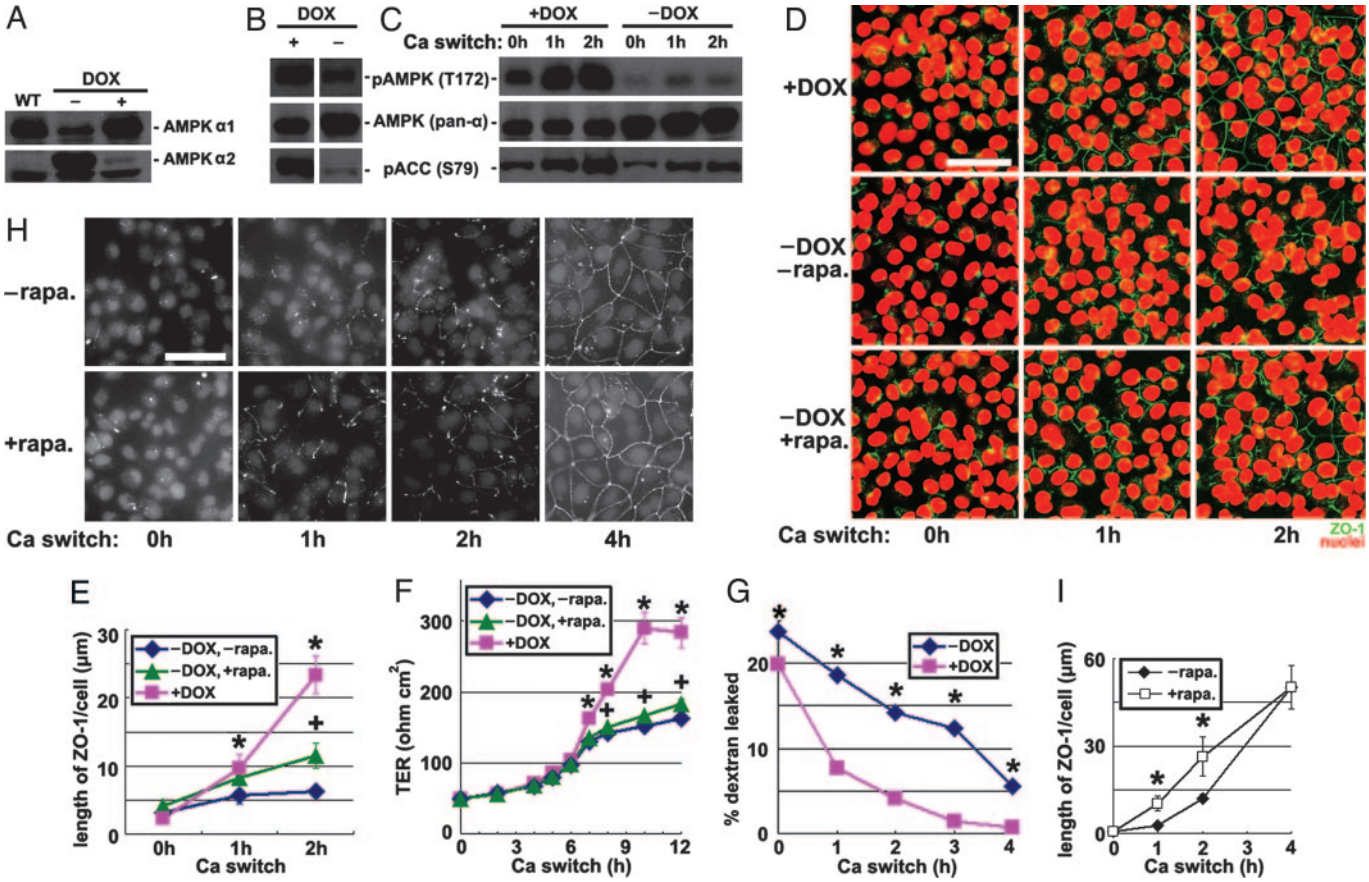

Fig. 3. Expression of DN AMPK delays tight junction assembly. (*A*) Lysates from untransfected Tet-Off MDCK cells (WT), as well as from K45R AMPK α2 transfected cells cultured in the presence or absence of doxycycline, were blotted with the indicated antibodies. (*B*) Lysates from transfected cells cultured in the presence or absence of doxycycline were blotted with the indicated antibodies. (*C*) Transfected cells cultured in the presence or absence of doxycycline were subjected to a calcium switch. Lysates from indicated time points after the switch from LCM to HCM were blotted with the indicated antibodies. (*D*) Localization of ZO-1 in transfected cells subjected to a calcium switch in the presence or absence of doxycycline or in the absence of doxycycline but with rapamycin treatment. (Scale bar: 30 μm.) (*E*) Quantification of ZO-1 relocation to cell–cell junctions during the calcium switch. (*F*) TER of transfected cell monolayers subjected to a calcium switch under the indicated conditions. (*G*) Paracellular flux assay performed on transfected cell monolayers subjected to a calcium switch under the indicated conditions. In *E*–*G*, the asterisks denote significant differences between cells cultured in the presence vs. the absence of doxycycline; the crosses denote significant differences with (−DOX, +rapa.) vs. without rapamycin (−DOX, −rapa.) treatment by Student's *t* test ($P < 0.05$). (*H*) Localization of ZO-1 in wild-type MDCK cells subjected to a calcium switch with rapamycin absent or present in the HCM. (Scale bar: 30 μm.) (*I*) Quantification of ZO-1 relocation to cell–cell junctions in the absence or presence of rapamycin. The asterisks denote significant differences in the presence vs. the absence of rapamycin by Student's *t* test ($P < 0.05$).

latory role in junction assembly is mediated, at least in part, by its inhibitory effects on mTOR activity.

To test this hypothesis, we treated MDCK cells expressing DN AMPK with 20 nM rapamycin for 1 h before the calcium switch to inhibit mTOR and then examined the relocation of ZO-1 to cell–cell junctions during the calcium switch. We observed that with rapamycin treatment the ZO-1 relocation is significantly accelerated in the cells expressing DN AMPK. However, the accelerated relocation of ZO-1 was still significantly slower than that in the cells not expressing DN AMPK (Fig. 3*D Middle* and *Bottom* and *E*). These data demonstrate that rapamycin treatment partially rescued the delay of ZO-1 localization caused by DN AMPK expression.

In line with the partial amelioration of defects in tight junction morphology during the calcium switch, the establishment of TER is also partially rescued by rapamycin treatment. The TER level of DN AMPK-expressing cell monolayers treated with rapamycin before the readdition of calcium is modestly but significantly higher than that of those without rapamycin treatment. However, the level of TER in DN AMPK-expressing cells with rapamycin treatment is still significantly lower than the monolayers cultured in the presence of doxycycline, when the DN AMPK construct is not expressed (Fig. 3*F*).

To investigate further the participation of mTOR in the assembly of tight junctions, we compared the rate of calcium-induced tight junction assembly in the presence or absence of rapamycin. If the direct inhibition of mTOR activity by rapamycin accelerates the assembly of tight junctions in wild-type MDCK cells, it would further support the involvement of the mTOR pathway in the regulation of tight junction assembly. We treated MDCK cells with 20 nM rapamycin for 1 h before the calcium switch and monitored the time course of the relocation of ZO-1 to the cell–cell junctions during the calcium switch, with rapamycin present in the HCM. We observed that ZO-1 relocation is significantly accelerated at the 1- and 2-h time points after the readdition of calcium in the cells incubated in the presence of rapamycin, compared with those incubated in the absence of rapamycin. The relocation of ZO-1 appeared to be complete at the 4-h time point, and no difference was observed in the cells incubated in the presence vs. in the absence of rapamycin at that time (Fig. 3 *H* and *I*).

Discussion

In this study, we have shown that AMPK is activated during calcium-induced tight junction assembly. The level of AMPK phosphorylation and activity increased significantly following the readdition of calcium. As AMPK is commonly thought to respond to changes in the cellular energy status, it is perhaps surprising that the activation of AMPK induced by calcium switch is not attributable to changes in cellular ATP levels, which remain relatively stable during the calcium switch. Recent identification of calcium/calmodulin-dependent protein kinase kinase (CaMKK) as another upstream kinase for AMPK (32–34) suggests that an influx of calcium caused by increased extracellular calcium levels during the calcium switch might up-regulate CaMKK activity, which would then in turn activate AMPK. The specific trigger responsible for AMPK activation in response to the calcium switch remains to be investigated. Our data indicate, however, that AMPK participates in other physiological functions above and beyond those associated with responding to energy status.

In the present study we reported that the activation of AMPK by AICAR facilitates the assembly of tight junctions, as demonstrated by the accelerated ZO-1 relocation to tight junctions, increased TER, and decreased paracellular permeability. These observations do not by themselves directly establish a requirement for AMPK in the process of tight junction assembly. The correlation between up-regulated AMPK activity and facilitated tight junction assembly, however, suggests that AMPK activation plays a role in this process. We also observed in this study that when cells are incubated in LCM, ZO-1 was relocated to the cell periphery when AMPK is activated by AICAR. The relocation of ZO-1 to cell–cell junctions represents an important step in the initiation of tight junction assembly. Although it has long been thought that tight junction assembly depends on extracellular calcium, our study suggests that activation of AMPK is able to bypass the calcium requirement for the initiation of tight junction assembly. These observations are consistent with those obtained in a previous study, in which activation of LKB1 was shown to initiate the relocation of ZO-1 to cell margins in the absence of cell–cell contact (13). Taken together, these findings suggest that AMPK may be the effector through which activated LKB1 initiates the redistribution of ZO-1 to sites of incipient cell–cell contact. We also found, however, that the nascent tight junction fragments do not propagate into a continuous structure in LCM. These data suggest that extracellular calcium is essential for the maintenance and amplification of the cues generated by AMPK activation for tight junction assembly.

We found that the tight junction assembly was significantly inhibited in cells expressing DN AMPK. Given enough time, however, normal-appearing tight junctions could eventually be formed in these cells. It would appear that AMPK activation supports the initiation of tight junction assembly and that other factors, including extracellular calcium, are sufficient to drive the propagation and long-term maintenance of tight junction structure. In cells expressing DN AMPK, the initiation of tight junction assembly is compromised, as evidenced by the delay of ZO-1 relocation to cell–cell junctions, the failure to establish TER at early time points, and the increased paracellular permeability during the calcium switch. The eventual junction formation in cells expressing DN AMPK might be attributable to the small amount of native AMPK that was not replaced by DN AMPK, which could slowly initiate the assembly of tight junctions. Once initiated, the assembly of tight junctions might be maintained by other factors, so that eventually a complete and mature tight junction can be formed. Alternatively, it is possible that AMPK is not absolutely required to support junction formation. Other proteins with overlapping or redundant functions may be present and capable of substituting for AMPK when this enzyme is absent. In this context, it is interesting to note that the kinase domain of AMPK is similar to another substrate of LKB1, PAR1 (14), a protein reported to participate in regulating epithelial polarity (15).

We found that rapamycin treatment of the DN AMPK-expressing cells partially corrected the delayed tight junction assembly. AMPK negatively regulates mTOR, and rapamycin directly inhibits mTOR. Thus, the partial amelioration of the DN AMPK defect by rapamycin suggests that the regulation of tight junction assembly by AMPK occurs, at least in part, through the inhibition of mTOR. The mTOR pathway has been shown to be involved in epithelial-mesenchymal transition (35) and in regulating cyst formation (36) in culture and animal models of renal cystic disease. Taken together with our data, it would appear that mTOR has a potential role in regulating aspects of epithelial polarization. In this context it is interesting to note that rapamycin appears to be effective in slowing the growth of malignancies. It is possible that this therapeutic efficacy of rapamycin in treating cancer is attributable, at least in part, to the prevention of cells from losing polarity and junctional integrity.

The fact that the rapamycin effect is incomplete suggests that, besides mTOR, there must be other downstream pathways through which AMPK participates in regulating tight junction assembly. A likely candidate is the PAR pathway. Interestingly, AMPK shares mechanistic homologies with MARK, which constitute the human homologs of PAR1. AMPK and PAR1 share a highly conserved kinase domain and are both activated by LKB1-dependent phosphorylation on a threonine residue within the same regulatory loop, known as the T-loop (14). Several recent studies documented the participation of PAR homologs in regulating epithelial polarity (15–20). It is possible, therefore, that AMPK is involved in regulating tight junction assembly by participating in the PAR pathway. We looked for possible interactions between AMPK and PAR3/PAR6/aPKC complex. We failed, however, to detect stable binding between AMPK and this complex by *in vivo* coimmunoprecipitation from confluent MDCK cells (data not shown). This negative result does not, of course, rule out possible transient interactions or interactions that are tightly defined temporally. Further investigation needs to be performed before any firm conclusion can be drawn regarding the relationship between AMPK and the PAR3/PAR6/aPKC complex.

Materials and Methods

Plasmids and Constructs. The K45R AMPK α2 construct was kindly provided by Morris J. Birnbaum (University of Pennsylvania, Philadelphia, PA) and was subcloned into a pRevTRE vector (Clontech). Details of construct preparation are available upon request.

Cell Culture and Transfection. The maintenance of MDCK cells is described in ref. 37. The Tet-Off MDCK cell line was purchased from Clontech. The pRevTRE vector was transfected into Tet-Off MDCK cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Calcium Switch. MDCK cells were grown in α-MEM until confluency. Cells were then rinsed with Ca^{2+}-free S-MEM (GIBCO) and incubated in S-MEM supplemented with 5% dialyzed FBS (GIBCO) for 16 h before being switched back to α-MEM for the indicated times.

Immunofluorescence and Quantification of ZO-1 Staining. Cells on coverslips were fixed in cold methanol and then permeabilized in goat serum dilution buffer (16% goat serum/20 mM Na_3PO_4, pH 7.4/450 mM NaCl/0.3% Triton X-100). Cells were incubated for 1 h with anti-ZO-1 (Chemicon), followed by incubation with Alexa Fluor 488-conjugated anti-rat IgG (Molecular Probes).

Cells were visualized on a Zeiss LSM 410 confocal microscope. Contrast and brightness settings were chosen so that all pixels were in the linear range.

To quantify the average ZO-1 length per cell, four fields were randomly selected from each coverslip, and the total length of ZO-1 at cell junctions in each field was outlined manually, followed by measurement on ImageJ software (National Institutes of Health). Cell numbers were counted for each field by using propidium iodide (Molecular Probes) staining to reveal nuclei, and the mean ZO-1 length per cell was calculated.

TER Measurements. MDCK cells were plated into 24-well Transwell (Corning) filter inserts for the measurements. TER was measured with a DVC-1000 voltage-current clamp (World Precision Instruments). The TER was determined by voltage-clamping the monolayer to 20 mV, measuring the resulting current deflection, and applying Ohm's law. For each data point, six parallel Transwell filters were measured.

Paracellular Flux Assay. MDCK cells were plated into 12-well Transwell filter inserts for the assay. At indicated time points following calcium switch, 500 μl of Texas red-conjugated 70-kDa dextran (1 mg/ml; Molecular Probes) was added into the apical chamber, and three parallel aliquots were taken from the basal chamber after 30 min incubation at 37°C. The fluorescence emission at 640 nm was measured after excitation at 590 nm by using a fluorescent plate reader (BMG Labtech).

Western Blot Analysis. Cells were lysed on ice with kinase lysis buffer (250 mM sucrose/20 mM Tris·HCl, pH 7.4/50 mM NaCl/50 mM NaF/5 mM $Na_4P_2O_7$/1 mM Na_3VO_4/2 mM DTT/1% Triton X-100). Cell lysates were cleared by centrifugation (18,000 $\times$ *g* for 20 min at 4°C). Samples were resolved by SDS/8% PAGE and electrophoretically transferred to nitrocellulose for incubation with anti-pan-α-AMPK, anti-pAMPK (Cell Signaling), anti-pACC (Upstate), anti-α1-AMPK, or anti-α2-AMPK (gifts from D. G. Hardie, University of Dundee, Dundee, Scotland). Blots were then probed with peroxidase-conjugated species appropriate secondary antibodies and visualized with the enhanced chemiluminescence (Amersham Biosciences).

ATP Assay. Samples for ATP assay were prepared as described in ref. 38. ATP concentrations were determined by using the FL-AA Bioluminescent assay kit (Sigma).

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